Title – *HS6ST1* insufficiency causes self-limited delayed puberty in contrast with other GnRH deficiency genes

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Abstract

Context: Self-limited delayed puberty (DP) segregates in an autosomal dominant pattern, but the genetic basis is largely unknown. Although DP is sometimes seen in relatives of patients with hypogonadotropic hypogonadism (HH), mutations in genes known to cause HH which segregate with the trait of familial self-limited DP have not yet been identified.

Objective: To assess the contribution of mutations in genes known to cause HH to the phenotype of self-limited DP.

Design, patients and setting: We performed whole exome sequencing in 67 probands and 93 relatives from a large cohort of familial self-limited DP, validated the pathogenicity of the identified gene variant *in vitro* and examined the tissue expression and functional requirement of the mouse homolog *in vivo*.

Results: A potentially pathogenic gene variant segregating with DP was identified in 1 of 28 known HH genes examined. This pathogenic variant occurred in *HS6ST1* in one pedigree and segregated with the trait in the six affected members with heterozygous transmission ($p= 3.01 \times 10^{-5}$). Biochemical analysis showed that this mutation reduced sulphotransferase activity *in vitro*. *Hs6st1* mRNA was expressed in peri-pubertal wild type mouse hypothalamus. GnRH neuron counts were similar in $Hs6st1^{+/-}$ and $Hs6st1^{+/+}$ mice, but vaginal opening was delayed in $Hs6st1^{+/-}$ mice despite normal postnatal growth.

Conclusions: We have linked a deleterious mutation in *HS6ST1* to familial self-limited DP and show that heterozygous *Hs6st1* loss causes DP in mice. In this study, the observed overlap in potentially pathogenic mutations contributing to the phenotypes of self-limited DP and HH was limited to this one gene.

Introduction

Abnormal pubertal timing affects over 4% of adolescents and is associated with adverse health and psychosocial outcomes (1-3). However, in most patients with early or late onset of puberty, the underlying pathophysiology is unknown. Self-limited delayed puberty (DP) represents an extreme variant of normal pubertal timing and has been shown to cluster in families (4). Several groups have noted that DP segregates in an autosomal dominant pattern (4,5), suggesting mono- or oligo-genetic inheritance conferred by haploinsufficiency or a gain of function mutation. As such, pedigrees with familial DP represent an invaluable resource to investigate the genetic regulation of puberty onset. Significant insights into the genetic control of the hypothalamic-pituitary-gonadal (HPG) axis have come from the discovery of rare variants underlying conditions of GnRH deficiency, such as hypogonadotropic hypogonadism (HH) (6). Perturbed embryonic migration of GnRH neurons from nose to hypothalamus has been shown to cause HH in humans and in animal models (7,8). Alternatively, HH may arise through mutations that affect GnRH secretion or function in the presence of a normal GnRH neuron number in the hypothalamus (9).

In view of the possible overlap between the pathophysiology of DP and conditions of GnRH deficiency, a few studies have examined the contribution of mutations in genes that cause HH to the pathogenesis of DP (10-12). Potentially pathogenic variants in a small number of genes causing HH (*GNRHR*, *TAC3*, *TACR3*, *IL17RD* and *SEMA3A*) were identified by whole exome sequencing (WES) in a few cases of self-limited DP, including those with constitutional delay of growth and puberty (13). Moreover, evidence from WES in our own cohort supports the hypothesis that mutations in genes which influence GnRH neuronal migration or development can cause self-limited DP (14). In this study, we assessed the contribution of mutations in genes known to cause HH to the phenotype of familial self-limited DP, and have identified a heterozygous *HS6ST1* mutation as a novel cause of self-limited DP.

Methods

Patients

The patients selected for this study belong to a previously described and accurately phenotyped Finnish DP cohort, for which diagnosis is based on objective evidence of a delayed pubertal growth spurt rather than self-recall (15). Patients were referred with DP to specialist paediatric care in central and southern Finland from 1982-2004. All patients (n=492) met the diagnostic criteria for self-limited DP, defined as the onset of Tanner genital stage II (testicular volume >3 ml) >13.5 yrs in boys or Tanner breast stage II >13.0 yrs in girls (i.e. two SD later than average pubertal development) (16). Medical history, clinical examination, and routine laboratory tests were reviewed to exclude those with chronic illness. HH, if suspected, was excluded by spontaneous pubertal development at follow-up. Normal fertility has been demonstrated in affected family members (5). Families of the DP patients were invited to participate via structured interviews and using archived height measurement records. The criteria for DP in probands' family members were: 1) age at take-off; or 2) peak height velocity (PHV) occurring 1.5 SD beyond the mean, i.e. age at take-off exceeding 12.9 and 11.3 yrs, or age at PHV exceeding 14.8 and 12.8 yrs in males and females; or 3) age at attaining adult height more than 18 or 16 yrs, in males and females, respectively (15). Written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry, Hospital District of Helsinki and Uusimaa (570/E7/2003). UK ethical approval was granted by the London-Chelsea NRES committee (13/LO/0257). The study was conducted in accordance with the guidelines of The Declaration of Helsinki.

Genetic Analysis

Genetic analysis was performed in 67 probands with DP from those 67 families with the greatest number of affected individuals in our cohort (male n=57, female n=10, see Supplementary Figure 1), 58 affected family members (male n=36, female n=22) and 35 of their unaffected family members (male, n=13, female n=22). WES was performed on DNA extracted from peripheral blood leukocytes of these 160 individuals, using a Nimblegen V2 or Agilent V5 platform and Illumina HiSeq 2000 sequencing. The exome sequences were aligned to the UCSC hg19 reference genome. Picard tools and the genome analysis toolkit were used to mark PCR duplicates, realign around indels, recalibrate quality scores and call variants.

Variants were analysed and filtered for potential causal variants using filters for quality control, predicted function, minor allele frequency (MAF) and biological relevance (Figure 1). Filtering by MAF included only variants with MAF <1% in the 1000 Genomes database, the NHLBI exome variant server and the ExAC and gnomAD databases. Biological relevance filtering allowed prioritisation of variants according to our 'HH genes' list (Table 1), comprising genes known to be relevant to the phenotype of HH based on previously published studies and using pathway analysis with Genego MetaCore (Thomson Reuters). The segregation with trait filter retained only variants present in ≥ n-1 affected individuals (where n = number of affected individuals in a given pedigree) and not present in more than one unaffected individual (Table 1).

Targeted exome sequencing (Fluidigm) of the remaining candidate gene post-filtering was performed in a further 42 families from the same cohort (288 individuals, 178 with DP; male=106, female=69 and 110 controls; male=55, female=58, Figure 1), with filtering as in (14). Whole gene rare variant burden testing was performed post sequencing. Fisher's exact test was used to compare the prevalence of deleterious variants in our cohort with the Finnish population, using the ExAC Browser (Exome Aggregation Consortium (ExAC), Cambridge, MA: http://exac.broadinstitute.org, accessed September 2015). For each gene, all variants from the ExAC database with minor allele frequency <2.5%, predicted to be deleterious by both Polyphen-2 (17) and SIFT (18), were included in the analysis, with each family in our cohort represented by the proband only. A multiple comparison adjustment was applied post hoc using the Benjamini & Hochberg method (19), as detailed in (14). All variants identified were confirmed via Sanger sequencing.

In vitro assay of sulphotransferase activity

The human *HS6ST1* cDNA (IMAGE consortium accession ID BC099638) was obtained from Genecopoeia in a pReceiverM14a backbone (pReceiverM14a-WThHS6ST1). The p.Arg375His and p.Met404Val point mutations were introduced by PCR-mediated mutagenesis (Quickchange II, Agilent Technologies), using the following primers: *Hs6st1*_R375H_FOR 5'-GCCTGAGGAGCCACGAGGAGCGTCT-3', *Hs6st1*_R375H_REV 5'-AGACGCTCCTCGTGGCTCCTCAGGC-3' for p.Arg375His and *Hs6st1*_M404V_FOR 5'-CCCACCGAGGACTACGTGAGCCACATCATTG-3', *Hs6st1*_M404V_REV 5'-

CAATGATGTGGCTCACGTAGTCCTCGGTGGG-3' for p.Met404Val. Mutations were confirmed by Sanger sequencing. COS7 cells, at 90% confluency in 6-well plates, were transfected with 2 μg of wild-type or mutant plasmids using Fugene HD (Promega). After 48 h, cells were lysed with 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100. pH 7.4. The homogenates were stirred for 1 hr and then centrifuged at 10,000 × g for 30 sec at 4 °C to clear debris. HS6ST1-FLAG fusion proteins were isolated from the supernatant using an anti-FLAG M2 affinity chromatography kit (A2220, Sigma). The purity of the eluted protein was checked by Coomassie Brilliant Blue (Thermo Fisher) staining of SDS-page electrophoretic gels. Protein relative amounts were calculated by densitometric analysis of Western blots with mouse monoclonal M1 anti-FLAG antibody (1:1000 dilution, Sigma).

Sulphotransferase activity was measured using R&D Systems' colorimetric assay (EA003). Briefly, different amounts of purified HS6ST1 proteins were added to a mix of 0.2 mM 3'-phosphoadenosine-5'-phosphosulfate (PAPS, donor), 1 mM N-acetylglucosamine (NAcGluc, acceptor) and 10 ng/ml inositol monophosphatase 3 (coupling enzyme) in a total volume of 50 μl of reaction buffer. The elution product of mock-transfected cells was used as negative control. The reaction was allowed to proceed for 20 min at 37°C with inorganic phosphate (Pi) measured immediately after by the Malachite Green Method. Enzymatic activity was represented as percent of WT activity.

Animals

Animal procedures were performed in accordance with institutional and UK Home Office guidelines. The *Hs6st1*$^{+/-}$ mice have been described previously (20,21) and were maintained in a C57/Bl6J background by pairing with wild type partners. *Hs6st1*$^{+/+}$ and *Hs6st1*$^{+/-}$ female mice were checked daily for vaginal opening (VO) and weight after weaning, i.e. from 21 days of age (22).

Histological analyses

Peri-pubertal and adult mice were perfused with 4% formaldehyde in PBS and then brains and testis were dissected and post-fixed overnight at 4 °C. Testes were dehydrated, embedded in paraffin, microtome sectioned at 8 μm and stained with hematoxylin and eosin, as previously described (23). For *in situ* hybridisation (ISH) or immunostaining, brains were cryoprotected in 30% sucrose and

frozen in OCT compound (VWR). 12-µm thick serial sagittal and coronal cryosections were collected on Superfrost Plus slides (VWR) and incubated with anti-sense riboprobes for *Gnrh* (24) or *Hs6st1* (21). In some experiments, we combined ISH with GnRH immunofluorescence and used a different mouse *Hs6st1* probe, which was generated by PCR-amplification from brain cDNA using 5'-ACTGGACCGAACTCACCAAC-3' and 5'-AACTCAGTGAGGCCGAAGAA-3'as primers and cloning into the dual promoter vector pGEM-T easy (Promega). Probes were generated as described (25). For immunoperoxidase staining, mouse 25-µm mouse brain cryosections were incubated with Bloxall (Vector Laboratories) to quench endogenous peroxidase activity before incubation with GnRH primary antibody (1:1000 in PBS, Immunostar) followed by incubation with biotinylated goat anti-rabbit antibody (1:400 in PBS; Vector Laboratories). Immunostaining was developed with the ABC kit (Vector Laboratories) and DAB (Sigma-Aldrich), as described (24).

Image processing and quantification

Images were acquired either using a Leica DM5500B microscope equipped with a DCF295 camera and DCViewer software (Leica) and then processed with Photoshop CS6 and Illustrator CS6 (Adobe) or acquired on an Axioskop2 Plus microscope (Zeiss) equipped with a TCH-5.0 ICE digital camera (TiEsseLab). In some experiments, bright-field images of ISH patterns were converted to RGB colour mode for superimposition onto fluorescent images. GnRH-positive cells were counted on 50 25-µm sections through the entire MPOA of each animal. To compare the abundance of GnRH-positive neurites at the ME, we measured the pixel intensity of GnRH staining in 25-µm thick coronal sections through the entire ME.

Statistical significance

Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were evaluated by 2-tailed student t-test and considered significant at $p < 0.05$. Statistical analysis was performed using Prism (GraphPad Software).

Results

Exome sequencing of families with self-limited delayed puberty identified an *HS6ST1* variant

Whole exome sequencing of 67 informative families from our large cohort with self-limited DP

identified 20 rare (MAF <1%) and predicted deleterious variants in 12 genes from a list of known HH genes (Table 1). However, after filtering for segregation with trait, only *HS6ST1* (ENSG00000136720, gene identification number 9394) was retained as a candidate gene (Figure 1). One proband and his affected relatives from one pedigree sequenced carried a rare and likely damaging *HS6ST1* variant (NM_004807.2: c.1124G>A (rs182882999) p.Arg375His) which causes a non-conservative amino acid substitution in the coding sequence. This proband carried an additional potentially pathogenic variant in a known HH gene, *FEZF1* (NM_ NM_001024613.3: c.1010T>A, p.Iso337Lys). However, this variant was not found in any other affected individual from this family and was, therefore, discounted due to lack of familial segregation.

The rare heterozygous missense variant *HS6ST1* (p.Arg375His) is predicted to be deleterious to protein function with 5/6 prediction tools, as the affected amino acid residue resides in a coiled-coil domain that is highly conserved among species, as revealed by the PhyloP and GERP score and a multiple sequence alignment (Supplementary Figure 2 and Supplementary Table 1). This specific variant was present at low MAF in some public databases, but whole gene rare variant burden testing showed significant enrichment of *HS6ST1* rare predicted pathogenic variants in our cohort as compared to ethnically-matched controls from the ExAC database (adjusted p value = $3.01 \times 10^{-5)}$) (Supplementary Table 2).

After targeted exome sequencing, two additional variants in *HS6ST1* were identified in three further affected probands (NM_004807.2: c199A>T (rs202247387) p.Lys67* and c585G>A p.Trp195*). However, Sanger sequencing did not validate the existence of these variants. No further rare, potentially damaging and trait segregating variants in *HS6ST1* were identified by targeted sequencing.

Pedigree with a potentially pathogenic *HS6ST1* variant displayed an autosomal dominant inheritance pattern and classical self-limited DP.

The pedigree with the p.Arg375His variant has several family members with typical features of self-limited DP (Figure 2, panel A). The proband was first investigated for growth delay at 12.8 yrs, at which time his bone age was 11 yrs (Figure 2, panel B). Examination at this stage found him to be prepubertal, with bilateral testis volumes of 2 ml and no pubic hair development. His blood

biochemistry at that time showed a typical picture of functional HH. Spontaneous onset of puberty was observed around 14.3 yrs of age. Over the next 2.4 yrs, he achieved testis volume and testosterone levels within the normal adult range. His sister's age at menarche was 15 yrs. Both siblings had normal birth weight and birth length. Their father and paternal uncle and aunt also had DP with delayed pubertal growth spurt. All family members with DP had self-reported normal olfaction.

The HS6ST1 p.Arg375His mutant protein has reduced sulphotransferase activity *in vitro.*

In vitro analysis of sulphotransferase function of the p.Arg375His mutant HS6ST1 protein (Figure 2, panel C) showed reduced activity at 39% of the wild type (WT) HS6ST1 protein (mean ± SD: 39.81± 8.21, $p<0.001$). This reduction in enzymatic function is within the range seen with other pathogenic mutations in *HS6ST1* found in patients with HH (26), and similar to that of the p.Met404Val mutant HS6ST1 protein (mean +/- SD: 28.8+/-1.46). This observation is consistent with the *HS6ST1* p.Arg375His mutation as causative in self-limited DP.

***Hs6st1* is expressed within the adult hypothalamus and olfactory bulbs**

In the adult brain, GnRH neurons are scattered in a bilateral continuum between the olfactory bulb (OB) and the medial preoptic area (MPOA) of the hypothalamus, where most cell bodies reside (27). We found that *Hs6st1* is highly expressed in the granular, mitral and glomerular layers of the adult OB (Figure 3, panel A) and diffusely in the MPOA (Figure 3, upper panel in B). *Hs6st1* expression was also observed in the arcuate nucleus (ARC), which harbours neurons that regulate GnRH secretion (Figure 3, lower panel in B). However, *Hs6st1* mRNA did not localise to $GnRH^+$ neurons (Figure 3, panel C). These observations suggest that *Hs6st1* is expressed by hypothalamic cells that do not correspond to GnRH neurons.

Normal positioning and number of GnRH neurons in the MPOA of Hs6st-1 heterozygous mice.

To determine whether HS6ST1 is required for the normal development of the GnRH neuron system, we compared mice heterozygous for a *Hs6st1* null allele ($Hs6st1^{+/-}$) to their wild type littermates ($Hs6st1^{+/+}$). However, there were no gross differences in brain or OB anatomy between the genotypes

(Figure 4, panels A-B). Moreover, there were no obvious differences in the positioning and number of GnRH neurons in the MPOA (Figure 4, panels C-D; number of GnRH+ cells: *Hs6st1*$^{+/-}$ 341.2±24.2 vs. *Hs6st1*$^{+/+}$ 378.3±30.2, n=5 each; p=0.37). GnRH neurons in *Hs6st1*$^{+/-}$ mice also projected similarly to the median eminence (ME), the site where they normally release GnRH into the portal blood vessels of the pituitary gland; moreover, the pixel intensity of GnRH-stained neurites was similar in both genotypes (Figure 4, panels E-F; % stained neurite area: *Hs6st1*$^{+/+}$ 2.9±0.4 vs. *Hs6st1*$^{+/-}$ 3.1±0.3, n=5 each; p=0.09).

_Hs6st1_$^{+/-}$ mice show significantly delayed puberty onset

To determine whether heterozygous *HS6ST1* deficiency is sufficient to cause DP, we compared the timing of puberty in *Hs6st1*$^{+/-}$ and *Hs6st1*$^{+/+}$ female mice by identifying the day of VO, a proxy measurement for pubertal activation of the HPG axis in mice (22). We found that VO was delayed by on average 1.88±0.82 days in *Hs6st1*$^{+/-}$ compared to *Hs6st1*$^{+/+}$ females (Figure 5, panel A, left; postnatal day (P) of VO: *Hs6st1*$^{+/+}$ 30.2±0.7, n=12, vs. *Hs6st1*$^{+/-}$ 31.9±0.5, n=13; p=0.04; mice were pooled form 6 different litters). DP in *Hs6st1*$^{+/-}$ females was not due to overall delayed development, as they were not smaller than *Hs6st1*$^{+/+}$ littermate females around the time of VO (weight at P30: *Hs6st1*$^{+/+}$14.12±0.58 g, n=12, vs. *Hs6st1*$^{+/-}$13.67±0.58 g, n=13; p=0.29). Instead, and consistent with their DP and thus older age at VO in the *Hs6st1*$^{+/-}$ females, there was an insignificant trend towards greater weight at the time of VO in *Hs6st1*$^{+/-}$ compared to *Hs6st1*$^{+/+}$ females within the cohort examined (Figure 5, panel A, right; body weight on the day of VO: *Hs6st1*$^{+/+}$ 14.9±0.3 g, n=12, vs. *Hs6st1*$^{+/-}$ 15.5±0.3 g, n=13; p=0.06).

Despite DP, the fertility of young adult *Hs6st1*$^{+/-}$ males and females of both sexes appeared normal, because they produced litters without obvious delay and of normal litter size when paired to wild type mice at 4-6 months of age (litter size: *Hs6st1*$^{+/-}$ x *Hs6st1*$^{+/+}$: 7.2±0.95 pups per litter in 6 litters, vs. *Hs6st1*$^{+/+}$ x *Hs6st1*$^{+/+}$ 7.3±0.71 pups per litter in 7 litters; p=0.89; *Hs6st1*$^{+/-}$ mice were born at the expected Mendelian ratio). In agreement with the ability to father litters of normal size, testes size was similar in young adult *Hs6st1*$^{+/-}$ and *Hs6st1*$^{+/+}$ males (Figure 5, panel B; P35 testes length in mm: *Hs6st1*$^{+/-}$ 8.30±0.16, n=6, vs. *Hs6st1*$^{+/+}$ 8.31±0.17, n=8; p=0.97). Moreover, testes showed normal

organisation of germ cells and interstitial Leydig cells and contained a similar number of seminiferous tubules per area (Figure 5, panels B,C; number of seminiferous tubule number per mm^2: *Hs6st1*$^{+/+}$ 14.53±0.91 vs. *Hs6st1*$^{+/-}$ 14.69±0.62; n=5 each; p=0.88). Together, these findings show that HS6ST1 haploinsufficiency delays puberty in mice without compromising fertility in the adult.

Discussion

The inheritance of self-limited DP is under strong genetic influence, with clear autosomal dominant segregation in many families with or without complete penetrance (4), and thus represents a useful basis for the investigation of puberty genetics (5). However, only a few genes responsible for self-limited DP have been identified. In view of the possible overlap between the pathophysiology of DP and HH, screening self-limited DP patients for mutations in known HH genes appears a prudent strategy; however, minimal genetic overlap between the two conditions has been identified to date (13). Additionally, there have been some observed differences in inheritance patterns and penetrance between the two conditions (28). In HH, over 40 different genes have been identified and incomplete penetrance is often seen within pedigrees, leading to a wide spectrum of phenotypes (29-31). As the understanding of the genetic basis of both self-limited DP and HH improves, it is likely that genetic testing will be able to help establish a definitive diagnosis in adolescent patients presenting with delayed onset of puberty.

Here, we have identified a deleterious mutation, p.Arg375His, in the HH gene *HS6ST1* as the likely causal factor for self-limited DP in one pedigree from our cohort of patients with familial DP.

HS6ST1 mutations have been previously identified in up to 2% of patients with idiopathic HH (26,32), but have not previously been reported in pedigrees segregating with the trait of self-limited DP. Our *in vitro* analysis showed that the *HS6ST1* p.Arg375His mutation had reduced sulphotransferase activity, comparable to previously published mutations in this gene in patients with HH (26). Taken together, these results suggest a working model in which *HS6ST1* heterozygosity causes self-limited DP, whilst potentially *HS6ST1* homozygosity or a 'second-hit' in a separate gene, would produce the phenotype of HH.

Our studies in a murine model corroborate heterozygous *Hs6st1* deficiency as a cause of delayed pubertal timing without compromised fertility. Thus, *Hs6st1*$^{+/-}$ mice were born at normal Mendelian ratios without obvious defects in GnRH neuron or testes development, but females showed delayed VO. The question of sex bias in self-limited DP is an interesting one, as it is diagnosed in up to 83% of boys and 30% of girls presenting with pubertal delay (33-35). The underlying reasons for this gender difference are not clear. However, this difference may be due, in part, to ascertainment bias. Indeed, a previous study has shown a near equal sex ratio consistent with autosomal dominant inheritance when all of the affected individuals from our large cohort were examined, as opposed to the probands only (5).

In our murine model of *Hs6st1*$^{+/-}$ mice, we did not find any evidence of a sex difference in anatomical studies. In this study only females were examined for timing of pubertal onset (VO), as VO is a very robust marker of pubertal onset in female mice. Male mice were not assessed for the timing of puberty, but the adult male mice had normal testicular morphology including the presence of spermatogenesis. Normal spermatogenesis is an excellent marker in males for reproductive competence and excludes GnRH deficiency. In females mice the reproductive competence was assessed as fertility of the females. With these methods GnRH deficiency was excluded both in male and female *Hs6st1*$^{+/-}$ mice. Importantly, mice deficient in *Hs6st1* were not significantly smaller or lighter than their wild type littermates, excluding poor growth as the underlying cause for pubertal delay. The viability of *Hs6st1*$^{+/-}$ mice is in contrast to that of *Hs6st1* knockout (*Hs6st1*$^{-/-}$) mice, which could not be examined for puberty defects due to embryonic lethality (36).

Our expression studies in mice indicate that *Hs6st1* is expressed in several tissues relevant to normal GnRH neuron migration or function, including the OB and the MPOA, ARC and PVN of the hypothalamus. However, we did not identify any obvious abnormalities in OB morphology, GnRH neuron number in the MPOA or in GnRH neuron innervation of the ME in *Hs6st1*$^{+/-}$ mice. Instead, *Hs6st1* expression in the ARC and PVN, where kisspeptin neurons and tanycytes modulate GnRH secretion and function (37,38), raises the possibility that HS6ST1 haploinsufficiency affects the regulation of GnRH neuron activity or other relevant downstream pathways.

Hs6st1 is required for the function of *Anos1* (also known as *Kal1*) and *Fgfr1*, two genes that are required for normal HPG axis function (26). In a *C. elegans* model, *Hs6st1* regulates neural branching in concert with *Anos1* and *Fgfr1* (26). Moreover, *Anos1* is also able to enhance *Fgfr1* signalling in a HS-dependent manner in an immortalised human cell model of GnRH neurons, and it has been proposed that this interaction promotes olfactory and GnRH neuron development (39). However, *Hs6st1* mutations may also impact on the HPG axis via *Anos1*-independent pathways, as HS modifications of many different proteoglycans in the extracellular matrix have the potential to affect multiple signalling pathways (40) previously implicated in the neuroendocrine control of fertility (41).

In summary, we have identified a new pathogenic mutation in *HS6ST1* as the likely cause of DP in a pedigree from our extensive patient cohort, with no other pathogenic mutations in genes known to cause HH identified in our cohort. These findings suggest that, with the exception of a few genes like *HS6ST1,* the genetic background of HH and DP is either largely different or is due to mutations in as yet undiscovered genes. Our findings differ from those in previous publications, in which up to 14% of DP probands were reported to carry potentially pathogenic variants in HH genes (13). However, previous results have not been adjusted for segregation with the DP trait within families, nor have the variants identified in these previous studies been tested for pathogenicity, and thus prior estimates of HH mutation rates in DP patients may have been an over-estimation. In agreement, prior to adjustment for segregation, our results suggested that 17.9% of DP probands had potentially pathogenic variants in HH genes, highlighting the value of familial data in identification of causal variants in a condition such as DP. Although further pedigrees with familial DP need to be studied to identify additional causative genes and provide accurate estimates of the genetic HH and DP overlap, our findings provide evidence that perturbations in a single allele of a gene regulating the HPG axis is sufficient to cause self-limited DP. In contrast, available evidence suggests that more deleterious alterations in the same gene, or in combination with additional genes, are required to cause more severe HH phenotypes.

URLs

dbSNP, http:// www.ncbi.nlm.nih.gov/projects/SNP/; 1000 Genomes Project, http:// www.1000genomes.org/; UniProt, http://www.uniprot.org/; SIFT, http://sift.jcvi.org/; PolyPhen-2, http:// genetics.bwh.harvard.edu/pph2/; LRT, http://annovar.openbioinformatics.org/en/latest/user-guide/filter/; Mutation taster, http://www.mutationtaster.org/; FATHMM, http://fathmm.biocompute.org.uk/; Ingenuity Variant Analysis, http:// www.qiagen.com/ingenuity; Genego MetaCore, https://portal.genego.com/; Ensembl variant effect predictor, http:// www.ensembl.org/info/docs/tools/vep/; Annovar, http:// annovar.openbioinformatics.org/

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Figure Legends

Figure 1 – Flowchart of WES (whole exome sequencing) filtering strategy to identify *HS6ST1*. Whole exome sequencing was performed on DNA extracted from peripheral blood leukocytes of 160 individuals from our cohort (67 DP probands, 58 DP relatives and 35 controls). The exome sequences were aligned to the UCSC hg19 reference genome. Picard tools and the genome analysis toolkit were used to mark PCR duplicates, realign around indels, recalibrate quality scores and call variants. Potential causal variants were identified using filters for quality control, predicted functional annotation, minor allele frequency (MAF), biological relevance (i.e. 'HH gene' list) and segregation with trait (see methods and table 1 for further information on filtering criteria). Targeted exome sequencing using a Fluidigm array of one candidate gene was then performed in a further 42 families from the same cohort (288 individuals, 178 with DP and 110 controls). Variants identified via targeted re-sequencing were filtered using the same criteria as the whole exome sequencing data, with additional rare variant burden testing. Functional annotation of the variants is as described in methods. DP – delayed puberty.

Figure 2 – Pedigree and clinical details of a patient with a p.Arg375His mutation in *HS6ST1* that impairs sulphotransferase activity

A: Pedigree of the proband with the p.Arg375His mutation. Squares indicate male family members, circles female family members. Black symbols represent clinically affected family members, grey symbols represent family members with unknown phenotype, clear symbols represent unaffected individuals. The arrow labelled 'P' indicates the proband in the family. A horizontal black line above or adjacent to an individual's symbol indicates that they are heterozygous for the p.Arg375His mutation as identified by either whole exome sequencing or Fluidigm array, and verified by Sanger sequencing.

B: Clinical details of the proband with the p.Arg375His mutation. Height chart for the proband shows reduction in growth velocity from 12 yrs of age, with associated delayed bone age shown in red. The subject was prepubertal until 14.1 yrs of age. This was followed by spontaneous onset of puberty and subsequent development over 2 yrs to normal adult testosterone levels and testicular volume. T vol – testicular volume, G – Tanner genital stage, Ph – Tanner pubic hair stage, LH – luteinising hormone, FSH – follicular stimulating hormone, T – testosterone.

C: Sulphotransferase activity assay. The p.Arg375His mutation reduces *HS6ST1* sulphotransferase activity. Relative specific activity (mean +/- SD) of recombinant WT or mutant (p.Arg375His or p.Met404Val) HS6ST1 proteins are shown. All assays were done as three independent biological repeats (n=3 for each experiment) using equal amounts of protein. *** $p<0.001$

Figure 3. *Hs6st1* mRNA expression in GnRH neuronal territories on postnatal day (P) 35.

A: Coronal sections of adult mouse OB were labelled for *Hs6st1* by in situ hybridisation The squared box in the left image is shown at higher magnification on the right side.

B: Contiguous coronal sections of adult mouse hypothalamus representing the MPOA (upper panels) and the ME (lower panels) were labelled for *Gnrh* and *Hs6st1* by in situ hybridisation; arrowheads indicate the *Hs6st1* expression in the arcuate nucleus and ME.

C: *Hs6st1* ISH (green) of coronal sections from P35 MPOA followed by immunolabeling for GnRH (red) revealed no expression of H*s6st1* in GnRH-positive neurons (example of GnRH neuron is indicated with a solid arrowhead).

Scale bars: 125 µm (A, B upper panels), 50 µm (B lower panels), 25 µm (C). Abbreviations: OB, olfactory bulb; GL, glomerular layer; MI, mitral layer; GR, granular layer; MPOA, medial preoptic area; ME, median eminence; 3v, third ventricle; ARC, arcuate nucleus.

Figure 4 – *Hs6st1*$^{+/-}$ mice showed no defects in olfactory bulbs nor in the number and projections of GnRH neurons.

(A-B) Perfused adult *Hs6st1*$^{+/+}$ and *Hs6st1*$^{+/-}$ brains were dissected, photographed and showed similar olfactory bulb structure in both genotypes.

(C-D) Coronal sections of adult mouse MPOA were immunostained for GnRH; arrowheads indicate examples of GnRH neurons that are normally present both in *Hs6st1*$^{+/-}$ mice and WT.

(E-F) Immunostaining for GnRH in coronal sections of adult mouse ME shows that GnRH neurons project similarly to the ME in *Hs6st1*$^{+/+}$ and *Hs6st1*$^{+/-}$ mice.

Scale bar: 3 mm (A, B), 125 µm (C, D), 50 µm (E, F). Abbreviations: MPOA, medial preoptic area; ME, median eminence; 3v, third ventricle. Arrows demonstrate representative GnRH cell bodies.

Figure 5 - Peri-pubertal female *Hs6st1*$^{+/-}$ mice show delayed vaginal opening while young adult male *Hs6st1*$^{+/-}$ mice showed normal testes morphology.

(A) Age (left graph) and weight (right graph) at the time of the vaginal opening in female *Hs6st1*$^{+/+}$ (n=12) and *Hs6st1*$^{+/-}$ (n=13) mice. Values for littermates are shown in the same colour. * indicates $p<0.05$

(B-C) *Hs6st1*$^{+/+}$ and *Hs6st1*$^{+/-}$ mice have similar testes size (upper panels of B), normal spermatogenesis after H&E staining of paraffin sections (lower panels of B) and similar number of seminiferous tubules (C).

Scale bars: 3 mm (B upper panels), 25 µm (B lower panels).

635 **Tables**

Gene	No. of rare and predicated damaging variants filtered from whole exome sequencing data	
	Not Segregating with the DP trait	Segregating with the DP trait
ANOS1	0	0
AXL	3	0
CHD7	3	0
DAX1	0	0
FEZF1	3	0
FGF8	0	0
FGFR1	2	0
GNRH1	0	0
GNRHR	0	0
HESX1	0	0
HS6ST1	1	1
KISS1	0	0
KISS1R	0	0
LEP	0	0
LEPR	0	0
LHX3	1	0
LHX4	1	0
NELF	0	0
PCSK1	0	0
PROK2	0	0
PROKR2	2	0
PROP1	1	0
SEMA3A	0	0
SF1	0	0
SPRY4	1	0
TAC3	1	0
TAC3R	0	0
WDR11	1	0

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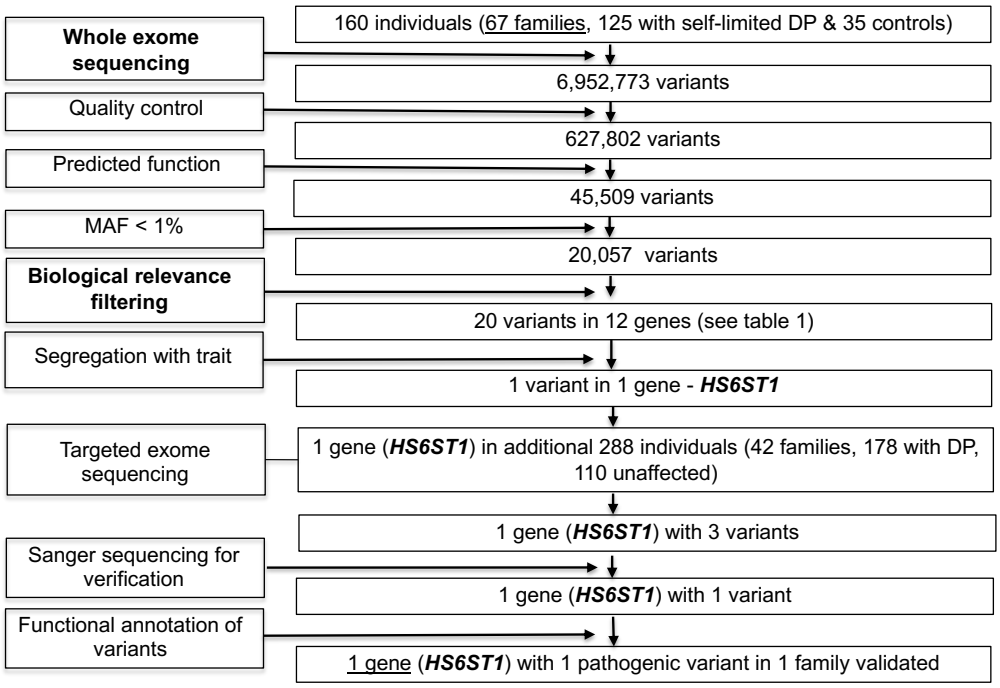
637 **Table 1 – HH gene filtering**

638 28 'HH genes', mutations in which have been identified as causal or potentially causal in patients
639 with GnRH deficiency, used for filtering post whole exome sequencing. Details given of number of
640 variants in these genes identified in 67 probands with DP after whole exome sequencing. Variants are
641 divided into those which were found to segregate with the DP trait within cohort pedigrees and those
642 that did not segregate.

643 **Figures**



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646 **Figure 1 – Flowchart of WES (whole exome sequencing) filtering strategy to identify *HS6ST1*.**
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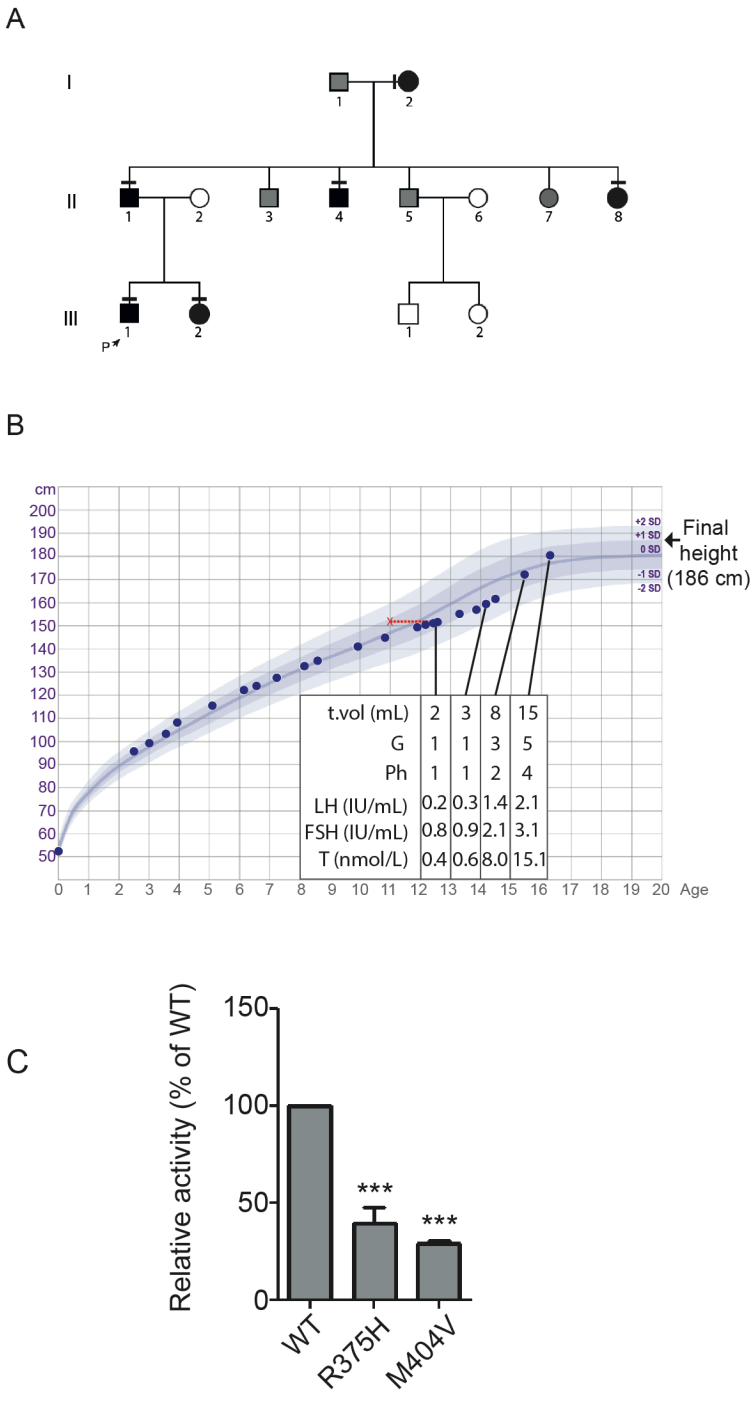

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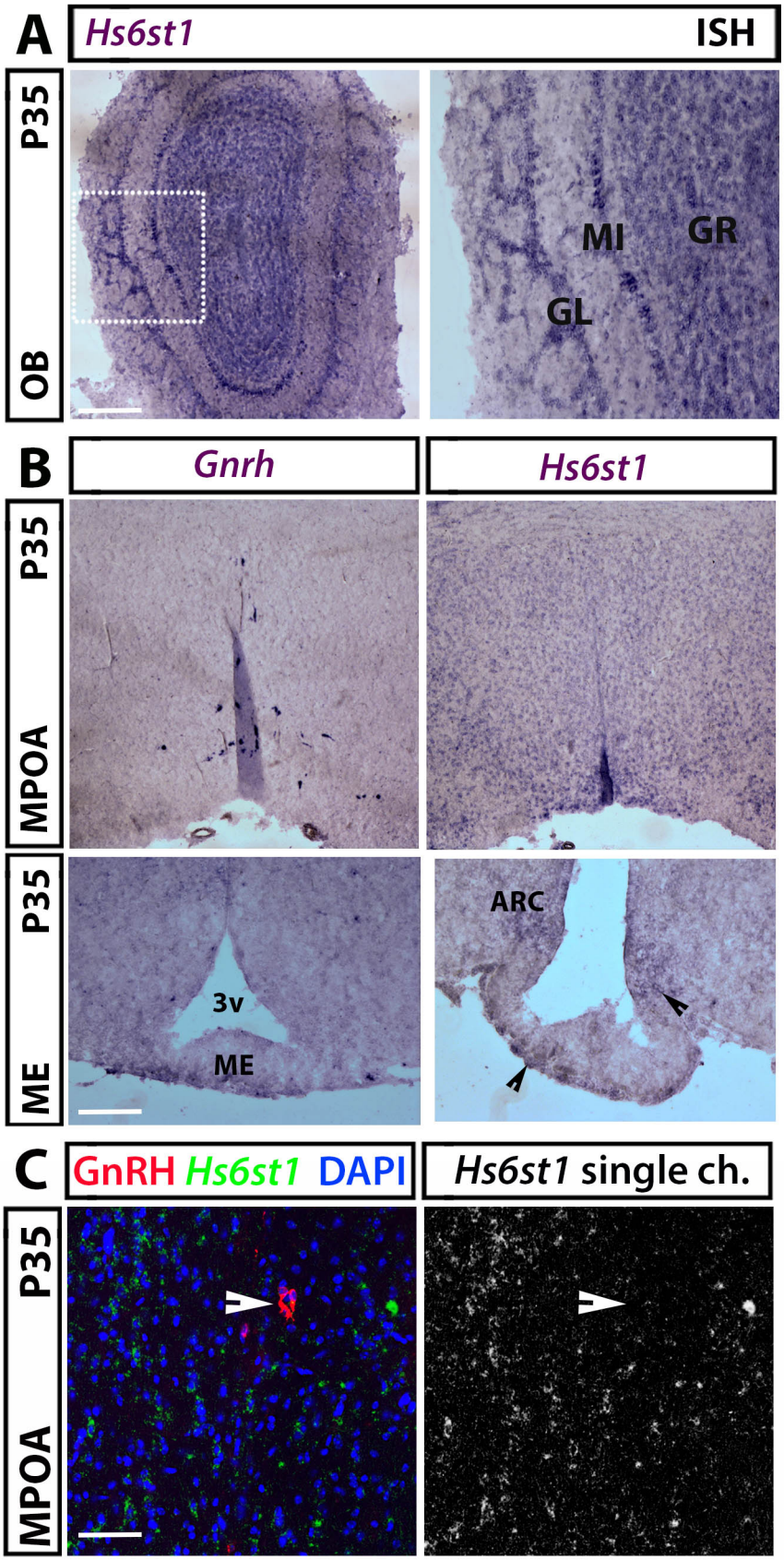

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655 **Figure 3. *Hs6st1* mRNA expression in GnRH neuronal territories on postnatal day (P) 35.**

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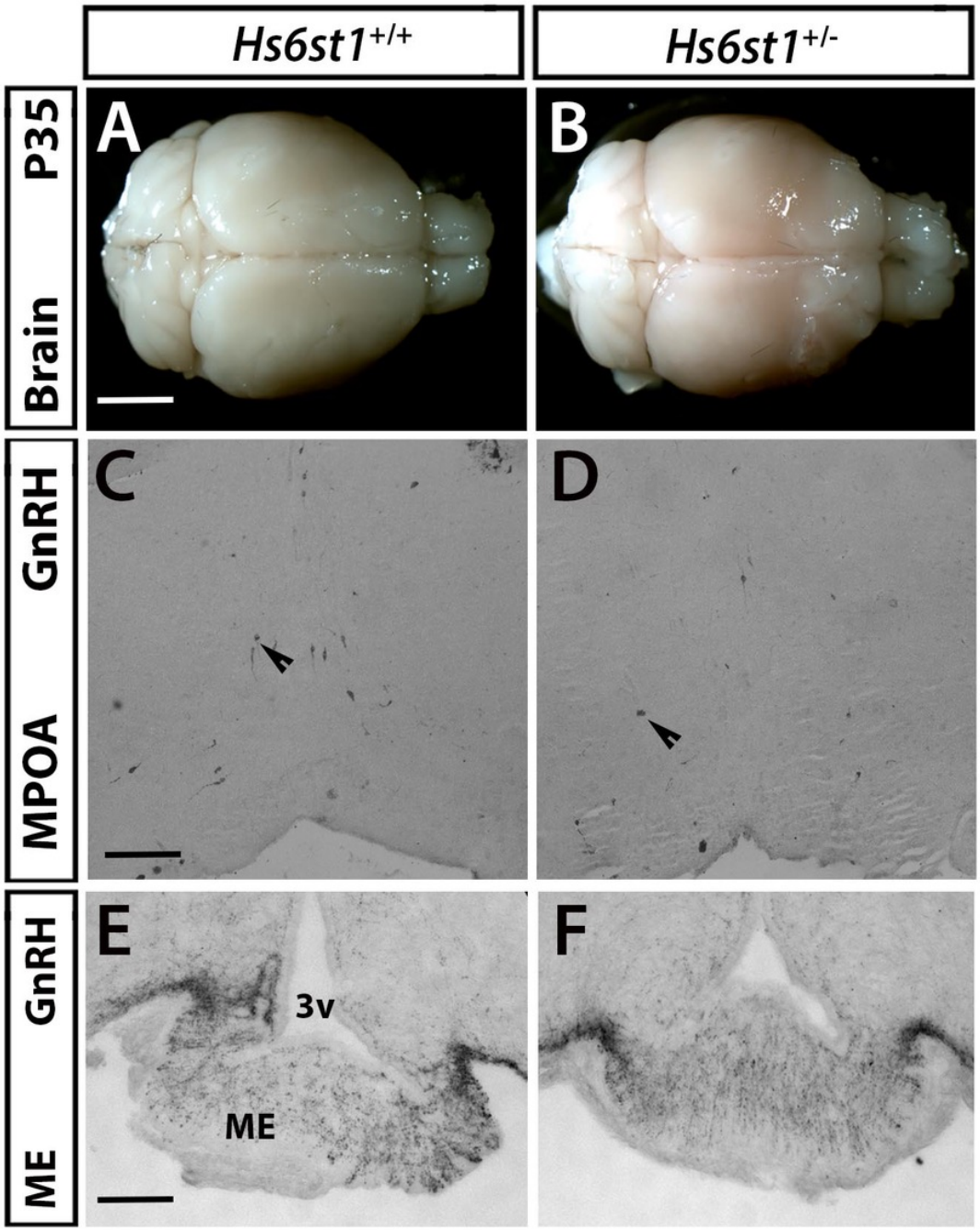

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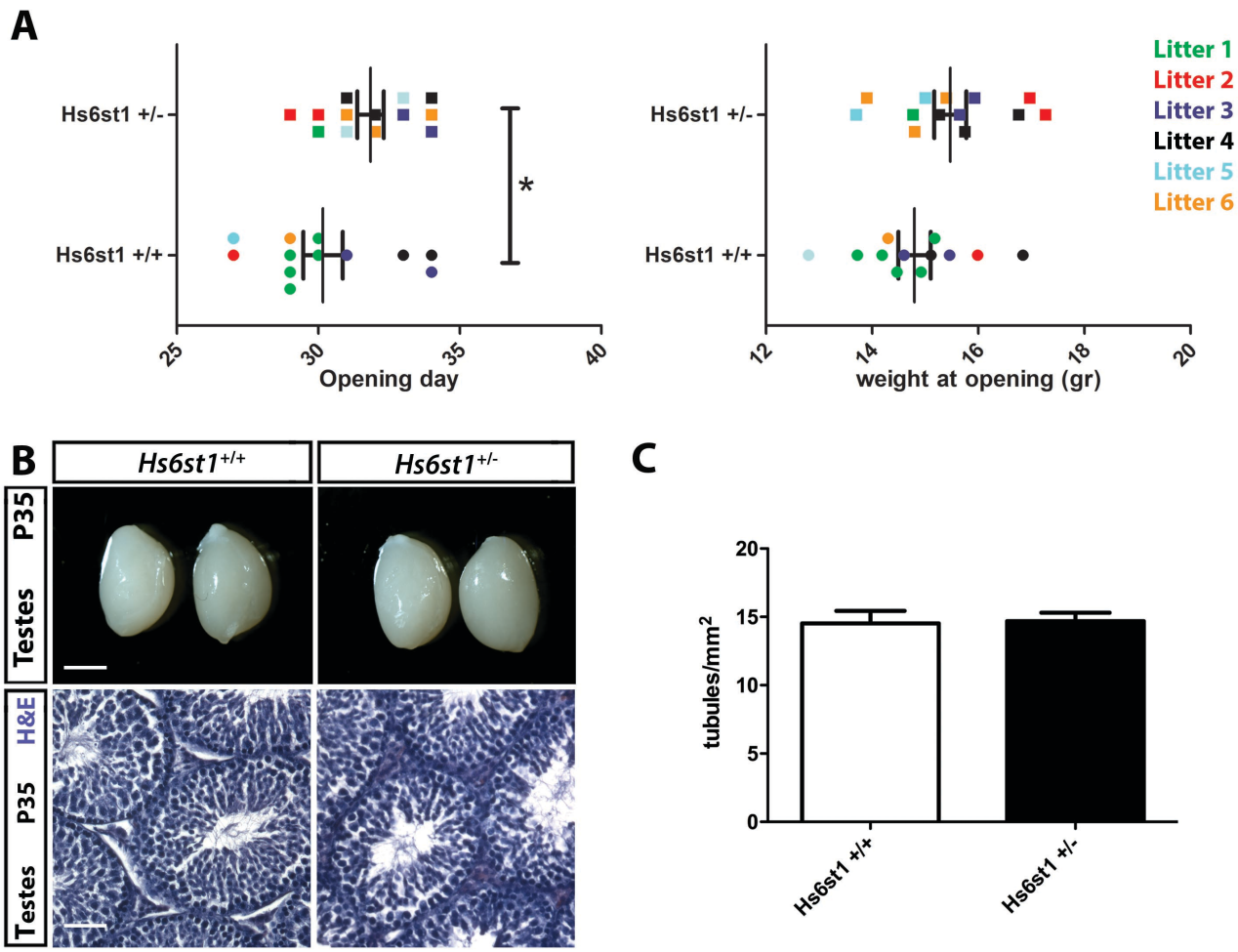

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